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Cytotoxic cyplasin of the sea hare, aplysia punctata, cDNA cloning and expression of bioactive recombinants

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Cytotoxic Cyplasin of the Sea Hare, Aplysia punctata, cDNA cloning and expression of bioactive recombinants

The present invention relates to a nucleic acid coding for a protein called "cyplasin" that shows a preferentially toxicity to autonomously growing mammalian cells. Cell death induced by this protein differs from both apoptosis and necrosis. An intracellular cell death which occurs when recombinantly preparing cyplasin in cell cultures can be avoided by removal of the secretion signal in the cyplasin sequence. This modification makes it possible to express the cyplasin in a mammalian cell culture which is preferable with regard to the glycosylation pattern of the obtained protein.

The invention concerns in more detail a

56-kDa protein isolated from the mucus of the European sea hare *Aplysia punctata* shows a preferential toxicity to autonomously growing transformed mammalian cells. Cell death induced by this protein differs from both apoptosis and necrosis. The cytotoxic effects are irreversible and become apparent at nanomolar concentrations in a cell type–dependent manner. In contrast, injection of micromolar concentrations into mice is tolerated without apparent negative consequences. Microsequencing of the 56-kDa protein released a peptide sequence whose corresponding nucleotide sequence was used as probe to screen *A. punctata* RNA-based cDNA and to select cDNA clones encoding polypeptides comprising the target peptide. Two closely related cDNA were detected. The cDNA encoding a polypeptide 558 aa in length was considered to reflect a *bona fide* clone encoding the cytotoxic protein. Its protein-coding section was recloned in vectors suitable for expression in *Escherichia coli*, in mammalian cells, and in insect cells, respectively. The *E. coli*–expressed polypeptide was biologically inactive. Transfected mammalian cells expressed a cytotoxic factor and died thereof as if treated with the genuine cytotoxic protein. In contrast, transfected insect cells, which proved to be much less sensitive when treated with the genuine protein, expressed the cytotoxic factor and continued to proliferate, allowing to establish stable insect cell lines expressing sufficient amounts of the cytotoxic factor for further characterization.

BACKGROUND OF THE TECHNOLOGY

Marine organisms represent an essentially unexploited reservoir for genes and metabolic products of potential biological and/or pharmacological interest [1-3]. So far, literature on natural products derived from marine organisms is dominated by low-molecular-weight compounds characterized by cytotoxicity. A number of such natural drugs are either clinically applied or under evaluation as potential anticancer drugs [1-3]. In contrast, reports on exploitable genes from marine organisms and their products are rare. The green fluorescent protein from the jellyfish *Aequorea victoria* may serve as an example for a gene of basic biological interest, which is widely used in biotechnology as reporter for studies on gene expression and protein localization in living cells [4]. The latter technology is also applied in the present study.

Sea hares appear to represent another species producing high-molecular-weight gene products of interest. Originally, the toxicity of the mollusc *Aplysia* was found to be due to low-molecular-weight metabolic substances deriving from algal diet [5]. However, cytolytic, antimicrobial, and antifungal activities could be detected in biochemical isolates of high molecular weight from the sea hares *Aplysia kurodai*, *A. juliana*, and *Dolabella auricularia*. Accordingly, it was suggested that these organisms might produce water-soluble gene-expressed biopolymers of pharmacological interest [5,6]. Furthermore, these biochemical investigations suggest that sea hares produce a number of closely related glycoproteins of different sizes and with different biological activities. First attempts to characterize these proteins on the sequence level led to the molecular cloning of one *A. kurodai*-derived cDNA, which showed significant sequence identities with the cDNA encoding a protein produced by the giant African snail *Achatina fulica* [7]. However, a clear correlation of the protein encoded by the cloned *A. kurodai* cDNA with any biological activity is missing. This is most likely due to the fact that the biologically active molecules are glycoproteins and that recombinant expression in *Escherichia coli* results in biologically inactive proteins.

The potential pharmacological value of *Aplysia*-derived proteins stimulated our approach to identify cytotoxic activities of the European sea hare *A. punctata* on the sequence level. A bioassay-guided fractionation of the secreted mucus of albumen glands released a 56-kDa glycoprotein, which showed cytotoxic effects on autonomously growing cells in nanomolar concentrations. Based on its cytotoxicity, its possible effects on neoplasia, and its origin *Aplysia*, the protein was termed cytotoxin. Microsequencing released an internal peptide whose corresponding nucleotide sequence was used as probe for the molecular cloning of two cDNA encoding closely related *A. punctata* proteins. A cytotoxic recombinant form of one of these variants is expressed in mammalian and in insect cells underlining the validity of the cloning approach and providing the basis for a potential application of this bioactive molecule.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to an isolated nucleic acid molecule encoding the protein cyplasin or a protein exhibiting biological properties thereof, being selected from the group consisting of

(a) a nucleic acid molecule encoding a protein that comprises the amino acid of Fig. 2;

(b) a nucleic acid molecule which hybridizes to a nucleic acid molecule specified for cyplasin

(c) a nucleic acid molecule the nucleic acid sequence of which deviates from the nucleic sequences specified in (a) or (b) due to the degeneration of the genetic code; and

(d) a nucleic acid molecule, which represents a fragment, derivative or allelic variation of a nucleic acid sequence specified in (a) to (c).

As used herein, a protein exhibiting biological properties of cyplasin is understood to be a protein having at least one of the biological activities of cyplasin.

As used herein, the term "isolated nucleic acid molecule" includes nucleic acid molecules substantially free of other nucleic acids, proteins, lipids, carbohydrates or other materials with which it is naturally associated. For example, an isolated nucleic acid molecule could be part of a vector or a composition of matter, or could be contained within a cell, and still be "isolated" because that vector, composition of matter, or particular cell is not the original environment of the nucleic acid molecule.

In a first embodiment, the invention provides an isolated nucleic acid molecule encoding the protein cyplasin comprising the amino acid sequence depicted in Fig. 2.

The nucleic acid molecules of the invention can be both DNA and RNA molecules. Suitable DNA molecules are, for example, genomic or cDNA molecules. It is understood that all nucleic acid molecules encoding all or a portion of cyplasin are also included, as long as they encode a protein with biological activity. The nucleic acid molecules of the invention can be isolated from natural sources or can be synthesized according to known methods.

The present invention also provides nucleic acid molecules which hybridize to the above nucleic acid molecules. As used herein, the term "hybridize" has the meaning of hybridization under conventional hybridization conditions, preferably under stringent conditions as described, for example, in Sambrook et al., Molecular Cloning, A Laboratory Manual, 2nd edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. Also contemplated are nucleic acid molecules that hybridize to cyplasin at lower stringency hybridization conditions. Changes in the stringency of hybridization and signal detection are primarily accomplished through the manipulation of formamide concentration (lower percentages of formamide result in lowered stringency), salt conditions, or temperature. For example, lower stringency conditions include an overnight incubation at 37°C in a solution comprising 6X SSPE (20X SSPE = 3M NaCl; 0.2M NaH_2PO_4; 0.02M EDTA, pH 7.4), 0.5% SDS, 30% formamide, 100 µg/ml salmon sperm blocking DNA, followed by washes at 50°C with 1 X SSPE, 0.1% SDS. In addition, to achieve even lower stringency, washes performed following stringent hybridization can be done at higher salt concentrations (e.g. 5X SSC). Variations in the above conditions may be accomplished through the inclusion and/or substitution of alternate blocking reagents used to suppress background in hybridization experiments. The inclusion of specific blocking reagents may

require modification of the hybridization conditions described above, due to problems with compatibility.

The nucleic acid molecules of the present invention also include molecules with sequences that are degenerate as a result of the genetic code.

In a further embodiment, the present invention provides nucleic acid molecules which comprise fragments, derivatives and allelic variants of the nucleic acid molecules described above encoding a protein of the invention. "Fragments" are understood to be parts of the nucleic acid molecules that are long enough to encode one of the described proteins. These fragments comprise nucleic acid molecules specifically hybridizing to transcripts of the nucleic acid molecules of the invention. These nucleic acid molecules can be used, for example, as probes or primers in the diagnostic assay and/or kit described below and, preferably, are oligonucleotides having a length of at least 15, preferably at least 50 nucleotides. The nucleic acid molecules and oligonucleotides of the invention can also be used, for example, as primers for a PCR reaction.

The term "derivative" in this context means that the sequences of these molecules differ from the sequences of the nucleic acid molecules described above at one or several positions but have a high level of homology to these sequences. Homology hereby means a sequence identity of at least 40 %, in particular an identity of at least 60 %, preferably of more than 80 % and particularly preferred of more than 90 %. These proteins encoded by the nucleic acid molecules have a sequence identity to

the amino acid sequence depicted in Fig. 2 of at least 80 %, preferably of 85 % and particularly preferred of more than 90 %, 95 %, 97 % and 99 %. The deviations to the above-described nucleic acid molecules may have been produced by deletion, substitution, insertion or recombination.

The nucleic acid molecules that are homologous to the above-described molecules and that represent derivatives of these molecules usually are variations of these molecules that represent modifications having the same biological function. They can be naturally occurring variations, for example sequences from other organisms, or mutations that can either occur naturally or that have been introduced by specific mutagenesis. Furthermore, the variations can be synthetically produced sequences. The allelic variants can be either naturally occurring variants or synthetically produced variants or variants produced by recombinant DNA processes.

Generally, by means of conventional molecular biological processes it is possible (see, e.g., Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, 2nd edition Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY) to introduce different mutations into the nucleic acid molecules of the invention.

For the manipulation in prokaryotic cells by means of genetic engineering the nucleic acid molecules of the invention or parts of these molecules can be introduced into plasmids allowing a mutagenesis or a modification of the sequence by recombination of DNA sequences. By means of conventional methods (cf. Sambrook et al., 1989, Molecular Cloning: A Laboratory Manual, 2nd edition, Cold Spring

Harbor Laboratory Press, NY, USA) bases can be exchanged and natural or synthetic sequences can be added. In order to link the DNA fragments with each other adapters or linkers can be added to the fragments. Furthermore, manipulations can be performed that provide suitable cleavage sites or that remove superfluous DNA or cleavage sites, preferably removal of the secretion signal. If insertions, deletions or substitutions are possible, in vitro mutagenesis, primer repair, restriction or ligation can be performed. As analysis method usually sequence analysis, restriction analysis and other biochemical or molecular biological methods are used.

The proteins encoded by the various variants of the nucleic acid molecules of the invention show certain common characteristics, such as enzyme activity, molecular weight, immunological reactivity or conformation or physical properties like the electrophoretical mobility, chromatographic behavior, sedimentation coefficients, solubility, spectroscopic properties, stability; pH optimum, temperature optimum.

The invention furthermore relates to vectors containing the nucleic acid molecules of the invention. Preferably, they are plasmids, cosmids, viruses, bacteriophages and other vectors usually used in the field of genetic engineering. Vectors suitable for use in the present invention include, but are not limited to the T7-based expression vector for expression in bacteria, the pMSXND expression vector for expression in mammalian cells and baculovirus-derived vectors for expression in insect cells. Preferably, the nucleic acid molecule of the invention is operatively linked to the regulatory elements in the recombinant vector of the invention that guarantee the transcription and synthesis of an RNA

in prokaryotic and/or eukaryotic cells that can be translated. The nucleotide sequence to be transcribed can be operably linked to a promotor like a T7, metallothionein I or polyhedrin promoter.

In a further embodiment, the present invention relates to recombinant host cells transiently or stably containing the nucleic acid molecules or vectors of the invention. A host cell is understood to be an organism that is capable to take up *in vitro* recombinant DNA and, if the case may be, to synthesize the proteins encoded by the nucleic acid molecules of the invention. Preferably, these cells are prokaryotic or eukaryotic cells, for example mammalian cells, bacterial cells, insect cells or yeast cells. The host cells of the invention are preferably characterized by the fact that the introduced nucleic acid molecule of the invention either is heterologous with regard to the transformed cell, i.e. that it does not naturally occur in these cells, or is localized at a place in the genome different from that of the corresponding naturally occurring sequence.

A further embodiment of the invention relates to isolated proteins exhibiting biological properties of the of cyplasin and being encoded by the nucleic acid molecules of the invention, as well as to methods for their production, whereby, e.g, a host cell of the invention is cultivated under conditions allowing the synthesis of the protein and the protein is subsequently isolated from the cultivated cells and/or the culture medium. Isolation and purification of the recombinantly produced proteins may be carried out by conventional means including preparative chromatography and affinity and immunological separations involving affinity chromatography with monoclonal or polyclonal antibodies.

As used herein, the term "isolated protein" includes proteins substantially free of other proteins, nucleic acids, lipids, carbohydrates or other materials with which it is naturally associated. Such proteins however not only comprise recombinantly produced proteins but include isolated naturally occurring proteins, synthetically produced proteins, or proteins produced by a combination of these methods. Means for preparing such proteins are well understood in the art. The proteins of the invention are preferably in a substantially purified form.

Molecular Cloning of Cypiasin-Encoding cDNA

cDNA prepared from total RNA of the albumen gland of *A. punctata* comprises more than one transcript encoding the peptide SGDYILIASYAD. Two cDNA were cloned encoding proteins, which diverge significantly in their carboxy-terminal sections but which comprise the target sequence (Figure 2). One of these cDNA encodes a protein of 558 aa residues with a molecular mass of 62.4 kDa (termed cypiasin-L), whereas another cDNA reflects a transcript encoding a shorter protein (421 aa residues, molecular mass 46.9 kDa, termed cypiasin-S). Moreover, PCR on total cDNA with cypiasin-L-specific primer pairs results in DNA fragments whose sequences diverge from those encoding cypiasin-L and cypiasin-S, respectively. Accordingly, mRNA appear to exist, which are neither identical with cypiasin-L nor with cypiasin-S. These sequence microheterogeneities suggest that *A. punctata* produces an unknown number of very similar, but not 100%, identical proteins that comprise the target sequence. On the basis of the available data, it cannot be decided whether these different mRNA and proteins derive from one single gene, e.g., by alternative splicing in combination with RNA editing, or whether there exists a cluster of very similar, but not 100%, identical genes.

Sequence Characteristics of the Proteins Cypiasin-L and Cypiasin-S Encoded by the Cloned cDNA

Biochemical data suggest (not shown) that the naturally occurring cypiasin is a glycoprotein. The cypiasin-L cDNA-derived amino acid sequence comprises five Asn-linked (N-X-S or N-X-T) glycosylation sites at positions N-151, N-271, N-401, N-416, and N-422 that is in agreement with the biochemical data. The glycosylation sites 1 to 4 are unchanged in the polypeptide derived from the cypiasin-S

cDNA, whereas the position N-422 is missing in the shorter sequence.

The N-termini start with a hydrophobic secretory signal sequence of high probability and a predicted cleavage site between aa residues 52 (Ser) and 53 (Ala). Accordingly, the molecular masses of the mature and expectedly functional proteins amount to 57.2 and 41.6 kDa, respectively. The calculated isoelectric points of these mature proteins are 5.54 (charge −13) for cyplasin-L and 6.20 (charge −5) for cyplasin-S.

Database searches with the nucleotide sequence released similarities with two other *Aplysia* sequences, namely *A. kurodai* albumen gland mRNA for aplysianin-A precursor (70.9% identities, D83255 [12]), and *Ac. fulica Ferussac* mRNA for achacin (52.2% identities, X64584 [7]).

Database searches with cyplasin subsequences released the amino acid sequences of the *Aplysia* species mentioned above and a number of protein sequences with longer strings of local identities or homologies. All the latter sequences belong to the class of monoamine oxidases. Table 1 shows alignments of one prominent cyplasin peptide string with subsequences of eukaryotic and prokaryotic monoamine oxidases. The significance of this finding remains to be elucidated; however, it is of interest to note that database searches with this and other cyplasin-typical strings released no significant hits with proteins from other classes.

Expression of Biologically Inactive Recombinants in E. coli

Recombinant expression of cyplasin-encoding cDNA sequences in the pQE/*E. coli* M15 system results in polypeptides, which are completely insoluble in buffers containing no detergents, and suspensions of such recombinantly expressed polypeptides could not exert any cyto-

Table 1. Database Searches with the pCyplasin-Derived Amino Acid Sequence Resulted in a Number of Hits with Sequences Reflecting Monoamine Oxidases.

Sequence	Accession Number	Organism
62 NIGVFEFCDRVGGRLFT 78	Cyplasin	*A. punctata*
+ V E DRVGGR FT	I51346	Rainbow trout
+ V E +RVGGR+ T	OXLA_CROAD	Crotalus
N+ V E +RVGGR +T	AOFA_BOVIN	Bovin
+ + V E D VGGR +T	AOFB_RAT	Rat
+ + V E DRVGGR +T	AOFA_HUMAN	Human
N+ V E DRVGGR +T	AOFB_HUMAN	Human
+ + FE +RVGGR+F+	T08202	Prokaryotic
+FE DR+GGR+ + +	T22714	Prokaryotic
+ VFE DRVGGR T	AOFH_MYCTU	Prokaryotic
+ + +FE + VGGR T	TR2M_AGRVI	Prokaryotic
++ V+E DR+GG+L+ +	TR2M_AGRRA	Prokaryotic
+ + + +E DRVGG+L++	A20966	Prokaryotic
+ + E R GGR+ T	E69899	Prokaryotic
++ + +E DRVGG+L+ +	TR2M_AGRT3	Prokaryotic
+ V E DRVGGR + +	PUQ_MICRU	Prokaryotic

Especially a motif between aa-positions 62 and 78 is frequently detected: A selection of aligned subsequences is displayed in the table below.

toxic effect when incubated together with cultured cells (not shown). This missing cytotoxic activity is suggestively due to incorrect folding and/or the absence of posttranslational modifications of the polypeptides expressed in the *E. coli* system.

Generation of Bioactive Recombinants in Mammalian Cells

In contrast, mammalian cells, e.g., HeLa S3 suspension cells, produce a cytotoxic factor when transfected with CMV vector-driven expression constructs specifying either cyplasin-L or EGFP-tagged cyplasin-L. This factor is not detectable in cultures of nontransfected cells nor in cultures transfected with constructs expressing the cyplasin-S version. The production of the cytotoxic factor is obvious because all cells of factor-producing cultures finally die in the typical manner that is observed when mammalian cells are treated with genuine cyplasin isolated from the mucus of *A. punctata*. Because only a fraction of cells in such cultures is transfected, it follows that the cytotoxic factor must be released from the producer cells with the consequence of cell death of producer and nonproducer cells. The release of the cytotoxic factor is well in agreement with the predicted secretory signal at the amino terminus of the cDNA-derived amino acid sequence (Figure 2).

Although this self-destructing system is not suitable to produce significant amounts of biologically active recombinants, it reveals the validity of the cDNA cloning approach and it indicates that the factor encoded by the cDNA with the longer insert shows the cyplasin-typical characteristics.

Recombinant Expression of Bioactive Cyplasin-L and Cyplasin-L to EGFP in Insect Cells

Insect cells (e.g., SF9) are known to be able to perform posttranslational modifications similar to mammalian cells. Because SF9 cells proved to be much less sensitive to genuine cyplasin preparations (not shown), they are especially suited to generate recombinant cyplasin in sufficient amounts for biological tests. Transfection of SF9 cells with pIZ vector-driven constructs specifying the expression of cyplasin-L or of EGFP-tagged cyplasin-L could not influence the proliferation rate of SF9 cells. Moreover, the medium of SF9 cells transfected with the construct specifying that cyplasin-L contained significant cytotoxic activity for mammalian cell cultures, which shows that the secretory signal of cyplasin-L is also functioning in insect cells.

In contrast, no cytotoxic factor was released from SF9 cells transfected with the construct specifying EGFP-tagged cyplasin-L. The cyplasin-L-EGFP fusion protein is clearly expressed in SF9 cells, as shown by EGFP-dependent fluorescence (Figure 3), but no significant amounts of the cytotoxic factor can be detected in the spent medium of spinner cultures. Interestingly, the Western blot shown in Figure 4 points to the deletion of the signal sequence in the cyplasin-L section of the fusion protein. This cleavage must occur in such a way that the truncated fusion protein remains cytosolic. Alternatively, retrograde translocation from the ER

to the cytosol has to be assumed. Such retrograde translocations have already been observed in other systems before [13-16].

However, the cytotoxic activity of the recombinantly expressed truncated cytlasin-L is maintained when fused to EGFP. The high-speed supernatant of homogenized cytlasin-L-EGFP-expressing SF9 cells was found to contain the factor that is cytotoxic to cultured mammalian cells. Consequently, stably transfected cytlasin-L-EGFP-expressing SF9 cell lines were generated by fluorescence-activated cell sorting, and fractions of the high-speed supernatants of such cultures contained the cytlasin-L-EGFP fusion protein (Figure 4) and exhibited the biological activities shown in Figure 5.

Characteristic Features of Cytlasin-Dependent Cytotoxicity

Proliferating mammalian cells exhibit characteristic time- and concentration-dependent morphological changes when treated with the biochemically isolated genuine cytlasin from the mucus of *A. punctata* (Figure 5). The cytotoxic effects of the genuine cytlasin become visible, e.g., in PtK cells, in less than 1 hour at 50 nM. For this cell line, the minimum cytotoxic cytlasin concentration is in the order of 2 nM; however, at this concentration, the cytotoxic effects appear foremost after 24 hours. Once induced, the cytlasin effect is irreversible and cell death is observed even if cytlasin-containing medium is replaced by fresh medium. Other cultured mammalian cells show lower (human skin fibroblasts, HSF) or even higher sensitivity (human melanoma cells, glia cells) (Figure 6).

The morphology of cytlasin-induced cell death is specific. The cells detach from the substratum, they shrink and disjoin from each other if grown as monolayer or in clusters, and occasionally they exhibit numerous small plasma vacuoles. Morphological changes of this type can also be observed in cells undergoing apoptotic cell death; however, typical indicators for apoptosis including nuclear fragmentation and exposure of phosphatidylserine on the outer membrane are missing (Figure 7). Similar forms of cell death have been described by Sperandio et al. [17] and were termed paraptosis.

Cytlasin exerts its cytotoxic effects only on cells in interphase. Mitotic cells are still able to complete anaphase and cytokinesis at a time when most interphase cells in the same culture already show the cytlasin-typical change in morphology (Figure 8). However, following completion of mitosis, these cells also die when reentering the interphase. Neither cell permeability nor the microtubular cytoskeleton nor intracellular Ca^{2+} levels are affected by cytlasin (not shown). Actin fibers, on the

other hand, react very sensitively to cytlasin. First signs of depolymerization appear already after 10 minutes; most of the actin cytoskeleton has disappeared after 30 minutes (Figure 9*b*) with few tangles of fibrous actin remaining around the nucleus (*cf.* Figure 9*c*). After a longer incubation of these cells with cytlasin, no more fibrous actin is found in the cytoplasm, with the exception of the cortical area (Figure 9*d – f*, *arrows*).

Evaluation of the Bioactive Recombinant Cytlasin-L-EGFP

A thorough side-by-side comparison of the biochemically isolated genuine cytlasin and the recombinant cytlasin-L-EGFP version meets the problem that the recombinant is, at present, only available on the level of enriched extracts. Although an exact quantitation is missing, so far, it is evident that the cytlasin-L-EGFP extracted from stably transfected SF9 cells exhibits cytotoxic activity, which is very similar to that induced by the biochemically isolated genuine cytlasin. Figure 5 presents side by side the effects of genuine cytlasin and recombinant cytlasin-L-EGFP on four different cell lines with established different sensitivities to genuine cytlasin. Using constant amounts of extracts from cytlasin-L-EGFP-expressing SF9 cells, it is obvious that HSFs are relatively insensitive to recombinant cytlasin-L-EGFP, which holds true also for the biochemically isolated genuine cytlasin. These cells only show a slight initial retraction and a weak tendency to shrink when treated either with genuine cytlasin (50 nM) or with the standard extracts containing the cytlasin-L-EGFP. Finally, they recover and continue to proliferate. Death of HSF cells is only observed at cytlasin concentrations in the order of 100 nM. In contrast, cells derived from a biopsy of a human melanoma exhibit significantly higher sensitivity when incubated with genuine cytlasin (1 nM) and with the standard extract. Melanoma cells treated either with the genuine cytlasin or with the recombinant cytlasin-L-EGFP show the typical cytlasin-induced retractions, the formation of vacuoles, and finally cell death. Other panels of this figure show glia cells from an established cell line derived from rat embryo cortices. These cells exhibit the highest cytlasin sensitivity of all cells studied so far. The typical cytlasin effect is observed at a concentration that is as low as 0.2 nM, and complete cell death is observed within a 5-hour observation period. The cells of the kangaroo rat line PtK are irreversibly damaged within 24 hours by incubation with 2 nM genuine cytlasin. A similar effect is observed after treatment with the standard extract. Prominent plasma vacuolisation and membrane changes are induced in these cells by genuine cytlasin as well as by recombinant cytlasin-L-EGFP.

Summarizing, these results show that the molecular cloning approach released a cDNA encoding a factor exhibiting cytotoxic activity similar to that detected in the secreted mucus of *A. punctata*, and that the cytotoxic effect

of the recombinant protein is not obliterated by its fusion to EGFP.

Target Site for Cytlasin Action

The exact mechanisms behind the cytotoxic effects of cytlasin and recombinant cytlasin are not yet elaborated. However, it is unlikely that the cells take up a protein of this size with the consequence of exerting negative intracellular influence. Long-term observations of cytlasin-treated cells indicate that the first signs of cytotoxic action occur at the outer cellular membrane, at a time when the internal cell morphology shows no anomalies. This observation suggests that cytlasin docking to the outer cellular membrane represents the trigger for a still unknown cascade of events that finally leads to cell death. This view is also in agreement with other observations. Mammalian cells transfected with expression constructs specifying cytlasin-L or EGFP-tagged cytlasin-L initially survive and they are able to produce the cytotoxic factor. However, they begin to exhibit the changed morphology as soon as the cytotoxic factor becomes detectable in the spent medium. This suggests that extracellular cytlasin is cytotoxic, whereas intracellular cytlasin is rather nontoxic.

Such a hypothesis was confirmed recently when, after removal of the secretion signal in the cytlasin sequence, mammalian cells were transfected with the modified construct. These cells expressed cytlasin, but continued to proliferate. Only upon homogenisation and subsequent purification did the cytotoxicity of cytlasin become apparent, killing now even the producing cells (Petzelt et al., unpublished).

Absence of In Vivo Toxicity of Cytlasin

In order to test if cytlasin showed cytotoxic effects also *in vivo*, either genuine or recombinant cytlasin was injected into three groups of mice. Group 1 consisted of 12 DBA2 mice, which were injected with a high concentration of cytlasin into the tail vein. The concentration used exceeded by far the concentration found to be toxic *in vitro*. Nevertheless, all mice survived, at least up to 4 weeks. The same result was obtained when in a second group 12 DBA2 mice were injected subcutaneously under identical conditions. They survived and no negative effects were found during the observation period. Finally, a third group (six mice) was injected into the tail vein using the recombinant cytlasin. Again all mice survived.

The results support previous suggestions pointing to cytotoxic substances of high molecular weight that are produced and secreted by *Aplysia* species [5,6]. Protein fractions from the secreted mucus of *A. punctata* show cytotoxic, and finally killing, activity when added to cells that grow independently of proliferation-controlling activities, e.g., in culture. One of these factors has been characterized on the peptide sequence level and it has been termed cyplasin. Interestingly, cyplasin shows a graded cytoxicity on cells in culture. It is highly cytotoxic to established cell lines, as shown for the glia cell line and PtK cells, as well as to many primary tumor cells, such as the human melanoma tested. HSFs show a significantly higher tolerance. Because other tumor cells tested are also highly sensitive (not shown), it appears that cyplasin is especially cytotoxic to established cell lines and to primary tumor cells. The different response of primary human fibroblasts is probably due to the fact that these cells cannot be considered as tumor cells although growing autonomously [18]. Accordingly, cyplasin might be useful for the specific elimination of nondesired cells in an organism, such as tumor cells.

Such a view is supported by preliminary *in vivo* experiments. In no case was a toxic effect of the injected cyplasin found when injected in normal mice, even when high concentrations of cyplasin were used. Presently, experiments with tumor-bearing animals are in progress to increase information on such preferential tumor cell cytotoxicity.

The natural source for cyplasin is limited; hence, its recombinant production appears to be a prerequisite for its potential application as an anticancer drug. In a first step, we searched for a cDNA, which could be considered to encode the protein with an apparent molecular mass of 56 kDa, which had been isolated by the bioassay-guided fractionation procedure. Using a subsequence of this protein as probe and conventional PCR and cDNA cloning techniques, we found that more than one *A. punctata* transcript comprises the subsequence used as specific probe. Two cDNA encoding polypeptides with diverging carboxy-termini could be identified on the sequence level. Moreover, individual cDNA clones showed slightly diverging nucleotide sequences when PCR products were cloned, which were prepared on the basis of complete *A. punctata* cDNA library template and primer pairs filling the coding regions of the cDNA identified in the first step. Actually, all individual clones investigated so far showed slightly different nucleotide sequences with the consequence of one or more amino acid exchanges in the corresponding polypeptide. It is highly unlikely that all these transcripts originate from different genes in *A. punctata*. Posttranslational processes like alternative splicing, differential polyadenylation, and RNA editing could result in transcripts encoding the different polypeptides.

At this stage, it is unknown whether the different polypeptides identified at the transcript level exhibit all identical functions. In this situation, it appeared worthwhile

to select only one cDNA species (encoding the protein termed cyplasin-L) and to investigate whether this sequence could encode a cytotoxic protein. The recombinant polypeptide produced in *E. coli* was found to be biologically inactive. However, eukaryotic cells transfected with constructs expressing this selected cDNA or this cDNA in fusion with the EGFP-encoding nucleotide sequence produced a cytotoxic factor that was not present in nontransfected cells nor in cyplasin-S–transfected cells. Insect cells (SF9) transfected with pIZ-driven expression constructs became especially useful. In this case, stably transfected cell lines could be established, which permitted the preparation of biologically active EGFP-tagged cyplasin-L in quantities sufficient to compare the biological activity of the recombinant protein with the material that can be biochemically isolated from the secreted mucus of *A. punctata*. The very similar morphological effects achieved by the biochemical isolate and by the recombinantly expressed protein suggest that the selected cDNA is a valid clone and that it encodes a protein presenting the cytotoxic principle of the genuine cyplasin of *A. punctata*. With the availability of bioactive recombinant cyplasin, it is now possible to evaluate its potential antitumor therapeutic value.

Further studies should reveal whether the cloned cDNA specifies the only cytotoxic protein among the slightly different transcripts mentioned above or whether other transcripts encode proteins that possess equal or even greater cytotoxic activity.

EXAMPLES

The following Examples are intended to illustrate, but not to limit the invention. While such Examples are typical of those that might be used, other methods known to those skilled in the art may alternatively be utilized.

Biochemical Isolation of Cytlasin

Mucus of albumen glands of the sea hare *A. punctata* can be obtained from animals during the spawning season when they come to the shore (around April on Ile d'Yeu). By gently squeezing the animal, the mucus (approximately 2.5 ml) is excreted as purple fluid, forming a gel when exposed to air. It is immediately diluted (1:1, vol/vol) with phosphate-buffered saline (PBS; 150 mM NaCl, 10 mM NaH_2PO_4, pH 7.2) and placed at 4°C. After 2 to 3 hours, the mixture becomes completely soluble. This step is followed by centrifugation at 10,000×*g*, 15 minutes, 4°C, to remove debris. The supernatant can be frozen and kept at −80°C without loss of activity. For further purification, the mucus is dialysed against 1000 vol of 50 mM MOPS, 1 mM dithioerythreitol, 0.5 mM EDTA, 5 mM KCl, pH 7.2 for 24 hours at 4°C. Protein fractions containing the cytotoxic activity were isolated by fractionated precipitation with ammonium sulphate. Cytotoxic activity was detected in precipitates collected between 33%/50% (pellet 1) and 50%/66% (pellet 2) saturation, respectively. Most of the cytotoxic activities were usually found in pellet 1. For cytotoxicity tests, pellets were dissolved in 300 µl of PBS, dialysed against the buffer described above. The most active fractions comprised protein(s) migrating as an essentially single band on a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel (Figure 1).

Identification of the SGDYILIASYAD Peptide in the Fraction of Cytotoxic Protein(s)

Material used for the microsequencing procedure was further purified by gel filtration (G-200 column; Sigma-Aldrich, Taufkirchen, Germany) in a buffer comprising 60 mM MOPS, 1 mM dithioerythreitol, 0.5 mM EDTA, 5 mM KCl, at pH 7.2. The dialyzed and lyophilized efflux was submitted to SDS-PAGE and blotted to a PVDF membrane (ProtoBlot; Applied Biosystems). Sections containing the region of interest were analysed by microsequencing procedures performed by WITA (Berlin, Germany).

Cytotoxicity Test

Aliquots from each pellet, dissolved in 300 µl of PBS, were tested for their toxic effects on autonomously growing cells. The term "autonomously growing cells" is used for all cells capable of proliferating *in vitro*, in contrast to cells proliferating within an organism. Routine tests were performed using the kangaroo rat cell line PtK2 and the human cell line HeLa. 4×10^4 cells were seeded in 24-well plates containing 500 µl of medium per well resulting in about 50% confluency after 24 hours. At this time, undiluted aliquots of the redissolved pellet(s) (5 µl) were added and cell cultures in parallel wells were supplemented with aliquots (5 µl) of serial dilutions.

Characterization of Cell Death Induced by Genuine Cytlasin

Morphological alterations of cells undergoing cytlasin-induced death were recorded by light microscopy. In addition, permeability changes of the plasma membranes were investigated by incubating the cytlasin-treated cells with the nonmembrane permeant compound H33257 (Sigma-Aldrich), 0.5 µg/ml, or propidium iodide (Boehringer Ingelheim, Germany), 1 µg/ml. Staining of nuclei was considered as indication for pathological permeability changes associated with necrosis or the final stages of apoptosis. For the visualization of the actin cytoskeleton,

times indicated, washed in prewarmed PBS, and fixed in ice-cold ethanol at −18°C for 10 minutes on ice. After several washes with PBS, cells were treated for 10 minutes in 0.5% bovine serum albumin (BSA) in order to reduce unspecific staining and incubated in FITC–phalloidin (Molecular Probes, Leiden, the Netherlands), diluted 1:300 in 0.5% BSA/PBS, for 45 minutes at room temperature. The unbound phalloidin was removed by several washes with PBS and the cells were viewed in a fluorescence microscope using the appropriate filters (ZEISS Axiovert 405). To differentiate the apoptotic form of death, cytiasin-treated cells were incubated in 5 μg/ml FITC-labeled Annexin V (Boehringer Ingelheim) for 20 minutes in Ca^{2+}-containing buffer and the presence of a potential phosphatidyl serine–Annexin complex was evaluated by fluorescence microscopy using appropriate filters [8]. For control, apoptosis was induced in cells by incubation with 0.2 μg/ml staurosporine for 3 hours. This treatment induced a clear translocation of phosphatidylserine to the outer face of the plasma membrane, thus becoming accessible to the FITC–Annexin [9]; the concentration of staurosporine, however, was sufficiently low to prevent the parallel staining of cell nuclei with propidium iodide.

A. punctata cDNA

Total RNA was isolated from albumen glands of the sea hare *A. punctata* by means of the Qiagen RNA isolation kit. The Clontech SMART II polymerase chain reaction (PCR) cDNA synthesis kit (K1052-1) was used to convert 100 ng amounts of total RNA into cDNA. First strand synthesis was primed with the modified oligo-dT included in the kit and primer extension was performed with the recommended RNase H⁻ point mutant reverse transcriptase (Superscript II; Gibco BRL). The SMART II oligo inducing the template switch at 5' ends was included in the first strand reaction. These reactions and PCR amplifications of first strand cDNA by means of the modified oligo (dT) and SMART II primers were performed according to the instructions of the producer of the kit.

Molecular Cloning of cDNA Encoding Proteins Comprising the Peptide SGDYILIASYAD

Amplified cDNA was used as a template and PCR reactions were primed with combinations of specific primers corresponding to the search sequence and with nonspecific primers, e.g., modified oligo-dT and SMART II, respectively. Amplification products was recloned in a pBluescript-derived T-overhang vector and sequenced. The validity of these sequences was verified by PCR reactions primed with oligo deoxynucleotides corresponding to sequences upstream and downstream of the specific SGDYILIASYAD-encoding primer. These probe-independent products contained the nucleotide sequence encoding the peptide SGDYILIASYAD. Sequences found upstream of SGDYILIASYAD-encoding sequence were unique, except for several base exchanges discussed in the text. In contrast, two 3' end sequences differing in length could be detected (L and S).

Fusion and Expression Constructs

The protein-coding sections were PCR-amplified with primers placing suitable restriction sites to the 5' and 3' ends of the amplification products. Following digestion with the corresponding restriction endonucleases, the products were either directly cloned into the expression vectors pcDNA3 (Invitrogen, Groningen, the Netherlands; for expression in mammalian cells), pQE30 (Qiagen; for expression in *E. coli*), pIZ/V5-His (Invitrogen; for expression in insect cells), or fused with the EGFP-encoding cDNA (Clontech) prepared in the *Xho*I/*Not*I sites of the pBluescript vector. Excision of the EGFP-tagged fragments and recloning in appropriate sites of the pcDNA3 vector or the pIZ/V5-His vector resulted in the corresponding cyplasin-EGFP expression constructs suitable for expression of fluorescently labeled fusion proteins in mammalian and insect cells, respectively.

Transfections and Recombinant Protein Expression

E. coli M15 cells were transformed with the pQE30 plasmids containing the cyplasin-L- and cyplasin-S-encoding inserts in frame with the His tag of the vector. The expressed His-tagged proteins were isolated by means of Ni-NTA agarose according to the protocol supplied by Qiagen.

Mammalian cells were transfected with the pcDNA3 plasmids containing either EGFP-tagged or nontagged cyplasin-L- and cyplasin-S-encoding inserts by means of the Effectene transfection kit (Qiagen). Cells transfected with constructs containing the insert encoding cyplasin-L-EGFP or cyplasin-L-EGFP could not survive longer periods. However, supernatants of such cultures contained the cytotoxic factor described in the text.

SF9 cells were transfected with the pIZ/V5-His plasmids containing either EGFP-tagged or nontagged cyplasin-L-encoding inserts using, in addition, the Effectene transfection kit (Qiagen). In contrast to mammalian cells, transfected insect cells survived. Expression was followed either by fluorescence microscopy of living cells or by testing of cytosolic extracts for the presence of a cytotoxic factor.

Stably Transfected SF9 Cells for Large-Scale Production of Cyplasin-L-EGFP

SF9 cells transfected with the plasmid pIZ/V5-His-cyplasin-L-EGFP were grown for 9 months as semi-attached cells at 26°C in TNM-FH insect medium (Applichem, Darmstadt, Germany) supplemented with 10% fetal calf serum, 5 mM Glutamax (Life Technologies, Karlsruhe, Germany), and 100 µg/ml zeocin (Invitrogen). The cell cultures were diluted 1:3 at 4-day intervals. The original transfection efficiency was approximately 10%; after a 3-month period, 5% of the cells remained fluorescent. The latter fraction was considered to be stably transfected. Cells of this fraction were separated by means of a fluorescence-activated cell sorter (Becton-Dickinson). Following a second sorting performed after 4 weeks, the resulting culture could be grown in spinner cultures up to several litres and

more than 90% of these cells expressed cyplasin-L–EGFP fusion protein.

Recovery of the Cytotoxic Factor from SF9 Cells Stably Expressing Cyplasin-L–EGFP

The EGFP-tagged cyplasin-L is not secreted into the medium of SF9. Routinely, 1 to 2×10^8 stably transfected SF9 cells were washed by suspension and centrifugation (1000×*g*, 3 minutes), once in PBS, and once in 50 mM MES, 1 mM EDTA, 5 mM KCl, 0.1% mercaptoethanol, pH 6.0. They were homogenized in 5 ml of the latter buffer. Homogenization and all subsequent steps were performed at 4°C. A protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) was present throughout the purification procedure. The homogenate was centrifuged (100,000×*g*, 60 minutes), and the supernatant was applied to a DEAE Cellulose column (DE52; Sigma-Aldrich) that had been equilibrated with the buffer described above. The column was washed extensively with the buffer used for equilibration followed by application of a NaCl gradient (0 to 200 mM). Eluted fractions were tested for the presence of the cytotoxic factor by addition of 100 µl of each fraction to indicator cells (PtK) growing in 500 µl of culture medium. If present, cytotoxic effects were observed after about 5 hours. Factor-containing fractions were eluted between 60 and 80 mM NaCl. Fractions with these characteristics were considered as "standard" extracts, and used for other biological tests.

Identification of Cyplasin-L–EGFP in Cytotoxic Extracts Isolated from Stably Transfected SF9 Cells

Protein fractions isolated as described above and exhibiting cytotoxic activity were concentrated and separated by 12.5% SDS-PAGE. Two identical samples (including a protein standard) were separated on the same gel. One section of the gel was stained using a silver-staining procedure; the other section was electroblotted (semidry blotting apparatus; Biometra, Göttingen, Germany) to a PVDF transfer membrane (Westran, Schleicher, and Schuell, Dassel, Germany). Buffer composition was 3.03 g of boric acid, 200 ml of methanol, 800 ml of H_2O, pH 9.0. Following blocking with BLOTTO [10], the membrane was incubated for 3 hours (26°C) with anti-GFP antibody (ABCAM, Cambridge, UK) diluted 1:2000 in PBS, pH 7.2, containing 0.1% BSA. After prolonged rinsing in PBS, immunodetection was performed by means of an alkaline phosphatase–coupled goat–antirabbit antibody (Dianova, Hamburg, Germany), which was applied for 3 hours at 26°C, diluted 1:12000 PBS, pH 7.2, containing 0.1% BSA. The blot was rinsed in PBS and placed into the staining solution consisting of 100 mM TRIS, 5 mM $MgCl_2$, 0.3 mg/ml nitro blue tetrazolium, 0.15 mg/ml 5-bromo-4-chloro-3-indolyl-phospate, pH 9.5.

Animal Experiments

DBA2 mice were injected with 300 µl (10 µM) of genuine cyplasin, either in the tail vein (group 1) or subcutaneously (group 2). Cyplasin had been dialysed before against a large

volume of PBS for 24 hours at 4°C and tested for positive cytotoxicity immediately before injection by incubating PIK cells with 10 nM cyplasin. Recombinant cyplasin was also dialysed against PBS, tested for positive cytotoxicity before injection, and 300 µl was injected into the tail vein. Mice were maintained under standard conditions and observed for 4 weeks.

Other Methods

Database searches and sequence analyses were performed by means of the HUSAR program package (DKFZ) that is a collection of sequence analysis tools based on the GCG program package developed by GCG. For the identification of the secretory signal sequence, we applied the McGeoch scan program [11]. DNA sequencing was performed by A. Hunziker (German Cancer Research Center) by means of an automatic DNA sequencer, model 373A (Applied Biosystems).

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Claims:

1. An isolated nucleic acid molecule encoding the protein cyplasin or a protein exhibiting biological properties of cyplasin selected from the group consisting of

(a) a nucleic acid molecule encoding a protein that comprises the amino acid sequence depicted in Fig. 2;

(b) a nucleic acid molecule which hybridizes to a nucleic acid molecule specified in (a);

(c) a nucleic acid molecule the nucleic acid sequence of which deviates from the ucleic sequences specified in (a) and (b) due to the degeneration of the genetic code; and

(d) a nucleic acid molecule, which represents a fragment, derivative or allelic variation of a nucleic acid sequence specified in (a) to (c).

2. A recombinant vector containing a nucleic acid molecule of claim 1.

3. The recombinant vector of claim 2 wherein the nucleic acid molecule is operatively linked to regulatory elements allowing transcription and synthesis of a translatable RNA in prokaryotic and/or eukaryotic host cells.

4. A recombinant host cell which contains the recombinant vector of claim 3.

5. The recombinant host cell of claim 4, which is a mammalian cell, a bacterial cell, an insect cell or a yeast cell.

6. An isolated protein exhibiting biological properties of cyplasin encoded by a nucleic acid molecule of claim 1.

7. An isolated protein exhibiting the biological properties of cysplasin wherein the normally occurring secretion sequence has been removed.

8. A recombinant host cell that expresses the isolated protein of claim 6 or 7.

9. A method of making an isolated protein exhibiting biological properties of cyplasin comprising:

(a) culturing the recombinant host cell of claim 6 under conditions such that said protein is expressed; and

(b) recovering said protein.

10. The protein produced by the method of claim 9.

11. A pharmaceutical composition comprising the protein of claim 6 or 7.

12. Use of the protein of claim 6 or 7 for preparing a pharmaceutical composition for treating cancer.

Abstract

A 56-kDa protein isolated from the mucus of the European sea hare *Aplysia punctata* shows a preferential toxicity to autonomously growing transformed mammalian cells. Cell death induced by this protein differs from both apoptosis and necrosis. The cytotoxic effects are irreversible and become apparent at nanomolar concentrations in a cell type-dependent manner. In contrast, injection of micromolar concentrations into mice is tolerated without apparent negative consequences. Microsequencing of the 56-kDa protein released a peptide sequence whose corresponding nucleotide sequence was used as probe to screen *A. punctata* RNA-based cDNA and to select cDNA clones encoding polypeptides comprising the target peptide. Two closely related cDNA were detected. The cDNA encoding a polypeptide 558 aa in length was considered to reflect a *bona fide* clone encoding the cytotoxic protein. Its protein-coding section was recloned in vectors suitable for expression in *Escherichia coli*, in mammalian cells, and in insect cells, respectively. The *E. coli*-expressed polypeptide was biologically inactive. Transfected mammalian cells expressed a cytotoxic factor and died thereof as if treated with the genuine cytotoxic protein. In contrast, transfected insect cells, which proved to be much less sensitive when treated with the genuine protein, expressed the cytotoxic factor and continued to proliferate, allowing to establish stable insect cell lines expressing sufficient amounts of the cytotoxic factor for further characterization.

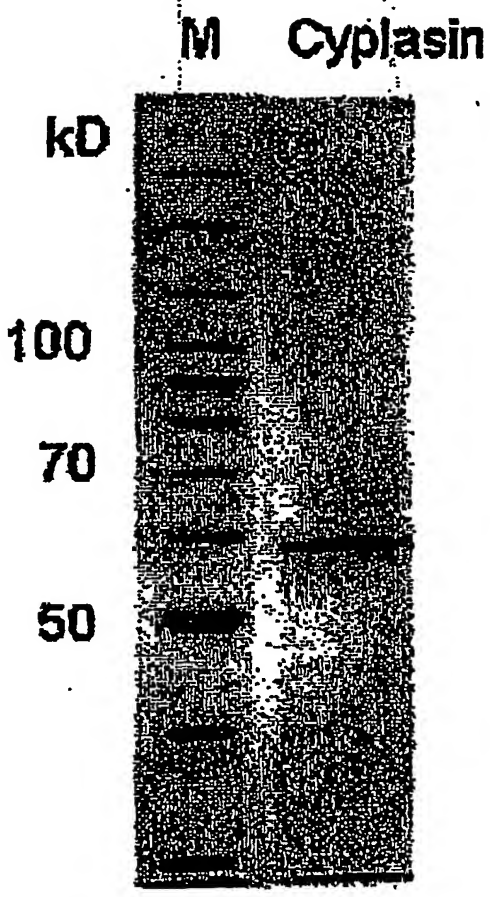

Figure 1. *SDS-PAGE of cyplasin isolated by a bioassay-guided fractionation of the secreted mucus of A. punctata. The figure shows a 12% SDS polyacrylamide gel loaded with the most active fraction (lane cyplasin). The proteinaceous material migrates with an apparent molecular mass of 56 kDa. Lane M is loaded with marker proteins.*

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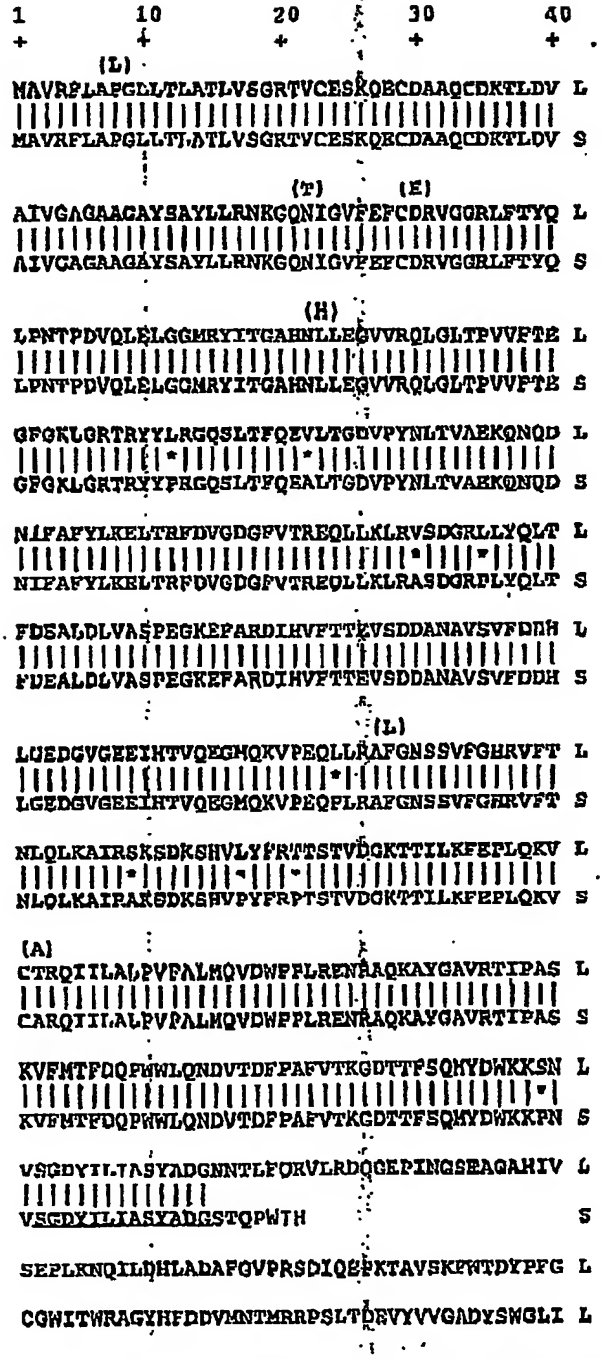

Figure 2. *Amino acid sequences of precursor proteins derived from A. punctata cDNA comprising the nucleotide subsequences coding for the (underscored) internal peptide SGDYILIASYAD. The upper sequence (558 aa residues) is derived from the nucleotide sequence of the cDNA encoding the polypeptide termed cypiasin-L and the lower sequence (421 aa residues) is derived from the nucleotide sequence of the cDNA encoding the polypeptide termed cypiasin-S. In addition to these clearly distinguishable transcripts, other mRNA may exist with additional differences. PCR with total cDNA as template and cypiasin-L-specific primer pairs releases sequences slightly differing from the cloned cypiasin-L and cypiasin-S encoding cDNA sequences. Amino acid exchanges detected by the PCR procedure are indicated in brackets. Asn-linked glycosylation sites are found at aa positions N-151, N-271, N-401, N-415, and N-422. The putative cleavage point of the secretory signal sequence is between aa 52 (S) and aa 53 (A).*

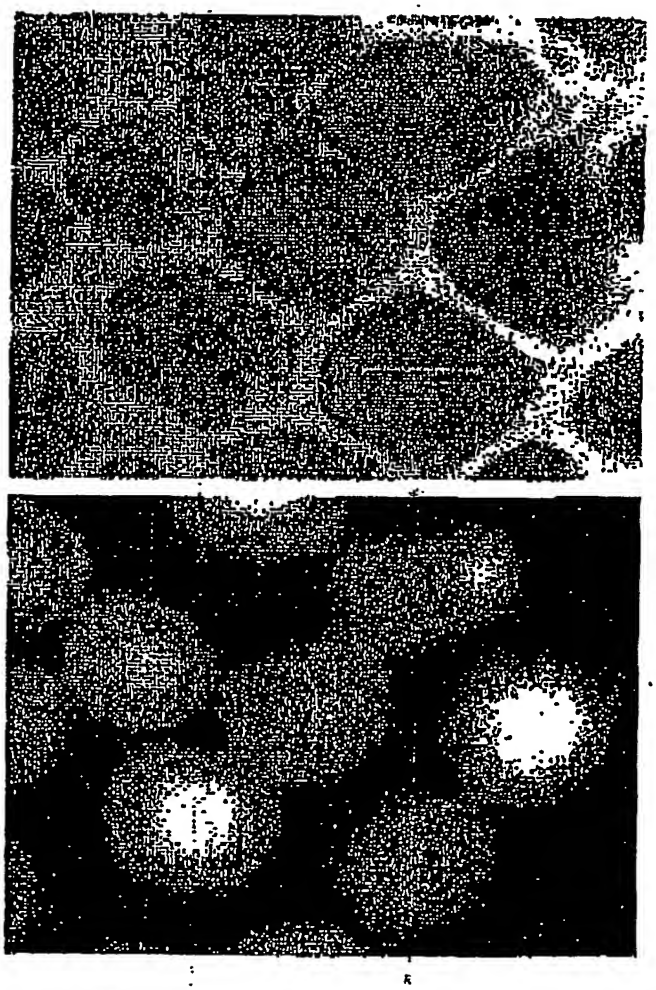

Figure 3. *Insect cells (SF9) transfected with the pIZ vector-driven construct expressing cytosin-L-EGFP. The upper panel shows SF9 cells in bright field and the lower panel shows the identical section in fluorescence mode (515 nm). Bar, 10 µm.*

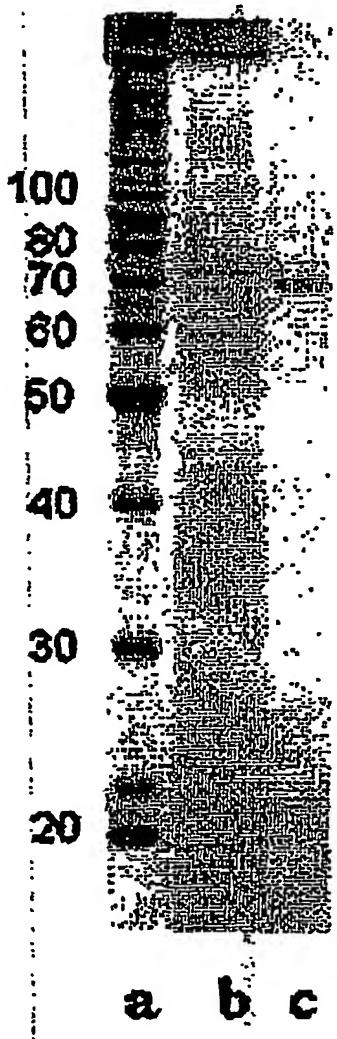

Figure 4. *Enrichment of the recombinant cytotoxin-L–EGFP fusion protein in cytotoxic protein fractions released from SF9 cells. Extracts containing the cytotoxic factor were prepared from SF9 cells expressing cytotoxin-L–EGFP as described under Materials and Methods section. Identical samples were separated on a 12% polyacrylamide gel. Polypeptides run on parallel gel sections, together with a protein size marker, were either visualized by a silver staining procedure or blotted to a PVDF membrane. The membrane was probed with an anti-EGFP antibody and immunocomplexes formed were visualized by means of an alkaline phosphatase–coupled second antibody. (a) Shows the protein size marker. (b) Shows the prominent polypeptides present in the extract. (c) Shows the antigen detected by the EGFP-specific antibody.*

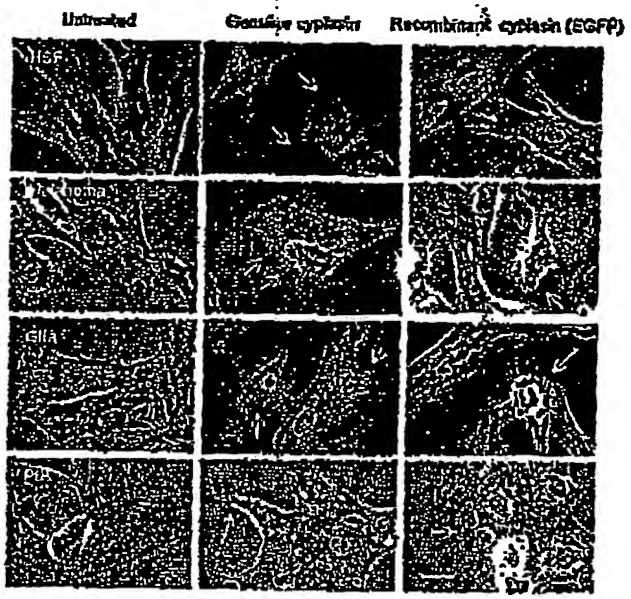

Figure 5. Cytotoxic effects of genuine and recombinant cytlasin-L-EGFP. Four different cell lines were treated for 5 hours with genuine cytlasin and with standard extracts (see Materials and Methods section) from SF9 cells stably expressing cytlasin-L-EGFP. Genuine cytlasin: Primary HSFs, incubated with 50 nM cytlasin. At this concentration, HSF cells show a slight but typical reaction that implies retraction of the cell membrane and partial detachment. Cell death is not observed at this concentration. The cells recover and continue to proliferate. Primary human melanoma cells derived from biopsies are more susceptible to the cytotoxic effect of cytlasin than HSF cells. After addition of cytlasin (2 nM), the cells show the typical cytlasin-induced membrane changes and finally die (arrows). Glia cells from a permanent cell line originating from the brain cortex of rat embryos are most sensitive when treated with cytlasin. Addition of 0.5 nM cytlasin is sufficient to induce cell death (arrows). Rat kangaroo PtK cells require 2 nM cytlasin to exhibit the morphology of dying cells. Recombinant cytlasin-L-EGFP: Standard extracts of recombinant cytlasin-L-EGFP (100 µl/500 µl medium) show, in parallel cultures, essentially identical and graded cytotoxic effects (arrows). Bar, 10 µm.

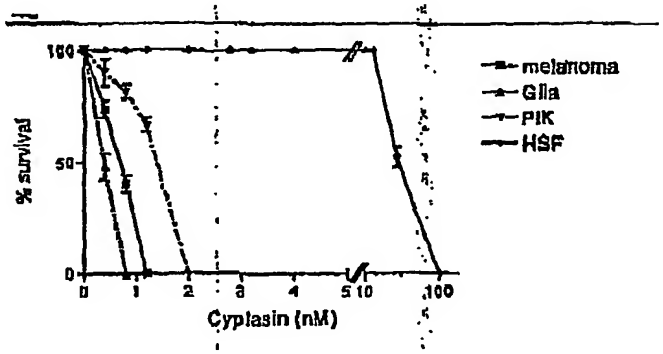

Figure 6. Dose–response curve of cytlasin for various cell lines. Glia cells are the cells most sensitive to cytlasin. Less than 1 nM cytlasin suffices to kill the majority of them. Primary human melanoma cells and PtK cells show also a high sensitivity to cytlasin, whereas HSFs are much more tolerant; only a dose as high as 100 nM cytlasin will kill these cells.

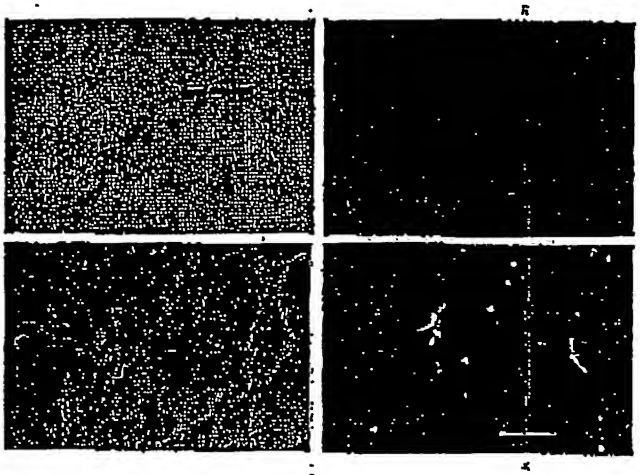

Figure 7. Apoptotic cell death induced by staurosporine and cell death induced by genuine cytisin. PtK cells were treated with 10 nM cytisin for 5 hours (upper panel), or with 1 µg/ml staurosporin for 3 hours (lower panel). The cells were stained with a mixture of FITC-labeled Annexin V and propidium iodide as described elsewhere in detail [8]. The FITC–Annexin V staining shows the characteristic translocation of phosphatidylserine from the inner to the outer side of the plasma membrane. No FITC–Annexin V staining is found in cytisin-treated cells that show the characteristic cytisin-induced morphological changes. Neither staurosporine nor cytisin permeabilizes the cells, which is revealed by missing propidium iodide staining of nuclei. Bar, 10 µm.

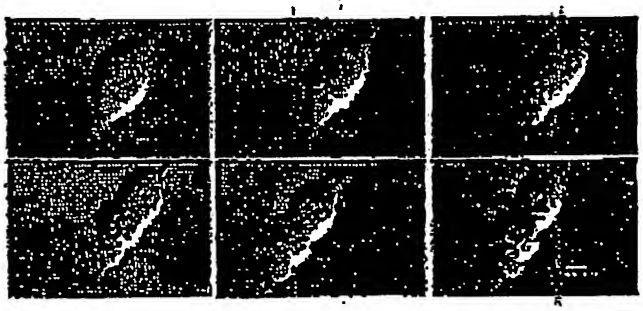

Figure 8. Anaphase progress of a PtK cell present in a culture treated for 1 hour with 2 nM genuine cytisin. From upper left to lower right: No interference is observed with the process of anaphase, which is terminating in an apparently normal cytokinesis. After entering interphase, this cell showed the typical cytisin-induced changes in morphology. Bar, 10 µm.

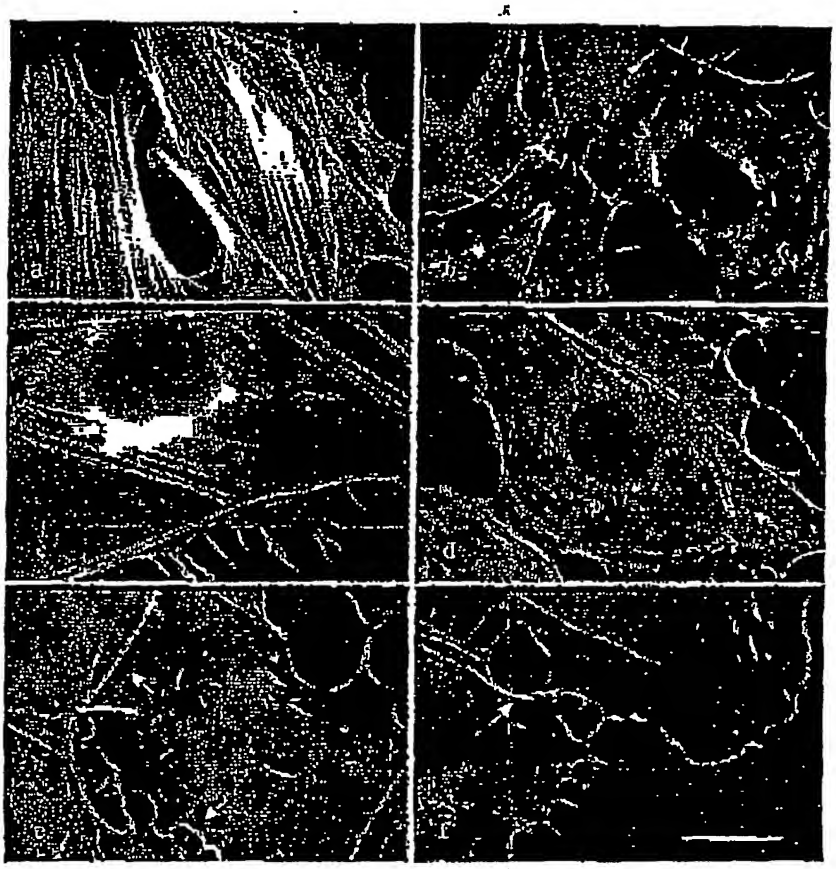

Figure 9. *Effect of cytosin on the actin cytoskeleton of human primary melanoma cells. Cytosin (10 nM) causes a fast depolymerization of actin fibers, with the exception of the cortical area where f-actin staining persists (arrows). (a) Untreated control; (b) 30-minute cytosin incubation; (c) 60-minute cytosin incubation; (d) 90-minute cytosin incubation; (e) 120-minute cytosin incubation; (f) 180-minute cytosin incubation. Bar, 10 μm.*